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Azole Preexposure Affects the *Aspergillus fumigatus* Population in Patients

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The relationship between the azole preexposure of 86 patients and the genotype, azole susceptibility, and *cyp51A* polymorphisms of 110 corresponding *Aspergillus fumigatus* isolates was explored. Isolates carrying serial polymorphisms (F46Y and M172V with or without N248T with or without D255E with or without E427K) had higher itraconazole MICs ($P = 0.04$), although <2 μg/ml using the EUCAST methodology, were associated with two genetic clusters ($P < 0.001$) and with voriconazole preexposure of patients ($P = 0.016$). Voriconazole preexposure influences the distribution of *A. fumigatus* isolates with selection of isolates carrying *cyp51A* polymorphisms and higher itraconazole MICs.

Azole resistance in environmental and clinical *Aspergillus fumigatus* isolates has become a major preoccupation since emerging azole resistance was described in The Netherlands (26) and the United Kingdom (9, 18). The prevalence of azole-resistant isolates was found to be 5.3% in The Netherlands in 2011 (28) and 5.8% in the ARTEMIS global surveillance study (22). The major mechanism involved in azole resistance is modification of the azole target, the Cyp51A protein (14 alpha-demethylase), with several mutations in the *cyp51A* gene responsible for various resistance phenotypes (19). Numerous polymorphisms have also been described in azole-sensitive isolates (14, 19). We took advantage of our single-center, hospital-based cohort study of consecutive *A. fumigatus* isolates prospectively collected from patients in the hematology department of our hospital between 2006 and 2009 (3) to study the impact of azole preexposure of patients on the isolates recovered by analyzing *cyp51A* gene polymorphism, *in vitro* azole susceptibility, and the distribution of genotypes based on microsatellite markers. We analyzed 110 isolates from 86 patients after excluding isolates with mixed genotypes ($n = 4$) and those with identical genotypes from the same patient ($n = 4$) to rule out the possibility of testing the same isolate several times. For each isolate, the whole *cyp51A* gene and promoter were sequenced as previously described (3) and genotyping was performed by using four previously described microsatellite loci (7).

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MIC susceptibility testing using Etest (AB bioMérieux) revealed that one isolate from an azole-naïve patient was itraconazole resistant (MIC, 16 μg/ml) and had its own genotype (3). The 109 remaining isolates were considered azole sensitive (itraconazole and voriconazole MICs of <2 μg/ml and posaconazole MICs of <0.25 μg/ml) (30). Unique single nucleotide polymorphisms (SNPs) were observed in three isolates: t173a, t1167a (N248K), and g1207t (D262Y). Several (8 to 12) synonymous and nonsynonymous SNPs were observed in 13 isolates (referred to here as sSNP isolates for serial SNP isolates) recovered from nine patients and classified into four groups on the basis of their *cyp51A* sequences (Table 1). After random selection, 10 of these sSNP isolates were compared to 10 isolates with the wild-type (WT) *cyp51A* sequence (GenBank accession no. AY048754) by using EUCAST methodology for itraconazole, voriconazole, and posaconazole sensitivity testing. The itraconazole MICs were significantly higher although <2 μg/ml and with <2-fold dilution differences for sSNP isolates (Table 1) than for WT isolates (Wilcoxon rank-sum test, $P = 0.04$; sum of ranks = 132.78 versus 78; U = 23.00), with no significant difference in the other azole MICs.

Genotyping of the 110 isolates revealed 95 different genotypes. Forty-nine genetically different isolates were recovered from 50 patients. Genetically identical isolates ($n = 25$) were collected from 23 patients (2 to 5 patients per genotype) who had or had not received azole therapy. Thirteen patients had iterative pulmonary isolates ($n = 36$; range, 2 to 7), some collected before or after azole therapy. For all of these 13 patients, the genotypes of the subsequent isolates were different from those of the first isolates, as already reported for pulmonary samples from hematology patients (4, 6, 12, 29).

The genetic variability of sSNP isolates compared with WT isolates was studied by the minimum spanning tree (MST) method (BioNumerics software v6.5) to group genotypes into genetic clusters (GCs) with the most stringent definition of GCs, i.e., tolerating only an allele difference in one marker, as already reported (8, 13, 16, 20, 23). Principal-component analysis (PCA) and unweighted-pair group method using average linkages (UPGMA) clustering analysis were also performed (MeV v4.6.1 software [24]). These three analyses highlighted the fact that sSNP isolates belong to distinct clouds (PCA) or clusters (MST, UPGMA tree) compared to WT isolates (data not shown). Among the sSNP isolates, two distinct GCs (GC1, $n = 6$; GC2, $n = 3$) and

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TABLE 1 Substitutions in the Cyp51A protein, EUCAST MICs, and *cyp51A* sequence genotypes of 13 *A. fumigatus* sSNP isolates recovered from nine patients

No. of sSNP isolates	Substitutions in Cyp51A protein[a]	MIC range (μg/ml)[f]			Patient no.	Gt[b]	Microsatellite marker[c]				GC[d]
		ITC	VRC	PSC			A	B	C	D	
8	F46Y, M172V, E427K	0.5–1	0.25–0.5	0.125	6	89	150	110	170	72	GC1
					1	90	154	110	170	72	GC1
					7	90	154	110	170	72	GC1
					8	91	154	110	172	72	GC1
					3	92	154	118	172	72	GC1
					9	93	158	110	170	72	GC1
					9	94	158	110	170	74	GC1
					5	79	130	114	172	72	S[e]
1	F46Y, M172V, N248T, E427K	0.5	0.25	0.0625	3	77	126	118	164	92	S[e]
3	F46Y, M172V, N248T, D255E, E427K	1	0.25–0.5	0.125	2	76	124	158	164	94	GC2
					4	78	126	158	164	94	GC2
					5	78	126	158	164	94	GC2
1	F46Y, M172V, N248T, D255E				1	26	104	128	118	96	S[e]

[a] The substitutions t18c, c283t, t489a (F46Y), c560t, c619t, g690a (M172V), a937g, and a1497g SNPs in the *cyp51A* gene were common to all isolates, while a1166c (N248T), c1188g (D255E), g1702 (E427K), and t1785c were inconsistently found.
[b] Gt, genotype. The values correspond to the genotypes of the isolates and are arbitrary.
[c] The values correspond to the lengths in base pairs of the PCR products of the four microsatellite markers (A to D) and are a function of the number of repeats of the microsatellite at each locus.
[d] The GCs were determined for all isolates using MST, PCA, and UPGMA clustering. GC1 and GC2 were genetically distinct from WT isolates using these three analyses.
[e] The isolates that had more than one allelic mismatch with the other isolates were considered singletons (S).
[f] ITC, itraconazole; VRC, voriconazole; PSC, posaconazole.

three genetically unrelated isolates were identified (Table 1). The sSNP isolates were significantly associated with GC1 and GC2 GCs compared with WT isolates ($P < 0.0001$, odds ratio [OR] = 225 [11.53 to 4,392], and $P = 0.005$, OR = 29 [2.7 to 303.7], respectively [Fischer exact test]).

To investigate whether azole preexposure affects *A. fumigatus* populations in patients, we analyzed azole preexposure at the time of recovery of each isolate. Twenty-six (24%) isolates were recovered from 14 patients undergoing voriconazole therapy for invasive aspergillosis (for 10 days to >2 years). Voriconazole preexposure was significantly associated with sSNP isolates (Fischer exact test; $P = 0.016$, OR = 4.3 [1.35 to 13.91]). Since different genotypes were recovered iteratively from 13 patients, some before and some after azole therapy, the patient-based analysis did not show any significant association between patients with sSNPs and voriconazole preexposure ($P = 0.159$, OR = 3.0 [0.65 to 13.80]).

Along with the association between voriconazole preexposure and sSNP isolates, we found higher itraconazole EUCAST MICs for sSNP isolates than for WT sequence isolates. Although this slight difference in MICs could be dismissed as nonsignificant since the MICs remained below the accepted threshold (2 μg/ml) for resistant isolates (30), this finding is consistent with a better tolerance of azole drugs by these isolates. This could explain why these sSNP isolates are less likely to acquire high-level azole resistance than WT isolates (15) despite the fact that they have been described with (19) or without (14, 19) hot-spot mutations responsible for high-level azole resistance. Our findings are consistent with those of Escribano et al., who reported a MIC of 2 μg/ml for three out of four such sSNP isolates that belonged to GCs distinct from those of WT isolates (14). It is unlikely that such serial polymorphisms appear in different individuals during medical azole therapy; a more plausible hypothesis is the environmental pressure exerted by the massive use of 14 alpha-demethylase inhibitors in agriculture (1, 2, 21, 25). This could explain the observed clonal expansion of sSNP isolates in distinct GCs, as reported for azole-resistant TR/L98H isolates (10, 22, 26). Since azole resistance can be associated with lower virulence in mice (5), there is a need to study the virulence of these sSNP isolates.

The treatment of invasive aspergillosis has changed considerably during the past few years, with numerous modifications in its management, especially the prescription of azoles as first-line therapy (17) or as prophylaxis (11, 27). Since voriconazole might select *A. fumigatus* isolates with specific Cyp51A polymorphisms associated with slightly better *in vitro* tolerance of itraconazole, our findings suggest that this phenomenon warrants continual surveillance.

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